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13. ABSTRACT (Maximum 200 Words)

The goal of this work is to develop a biomarker profile for prostate cancer risk, based on cell signaling proteins that serve as high-level biomarkers of cellular subsystems; e.g., tissue transglutaminase (TTGase), gelsolin, beta-catenin, thymosin beta-4, G-actin, E-cadherin, survivin, and purinergic receptor P_2X7. TTGase, cadherins, and G-actin are strong biomarkers of prostate cancer and may be strong biomarkers of field disease and premalignancy. The biomarker profile will identify individuals for prostate cancer prevention and rebiopsy, with high sensitivity and specificity, complementing serum PSA screening that captures 95% of prostate cancer cases with only 20% specificity.

Acquisition and fixation of prostate tissues are optimized. Prostate and bladder cell lines are being used for standardization purposes. Optimal labeling conditions including epitope retrieval, incubation periods and indicator systems are tested, analyzed, and compared. Quantitative Fluorescence Imaging Analysis (QFIA) allows precise quantification of high-level biomarkers in prostate tissues and cell lines. The observed down regulation of TTGase and beta-catenin in resected, cancer-bearing prostate glands suggests that high-level biomarkers of cellular subsystems could be effective biomarkers of early prostate cancer and premalignant disease.

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INTRODUCTION

Prostate cancer is the second leading cause of cancer deaths among men in the U.S. For the year 2004, the American Cancer Society estimated 230,110 new prostate cancer cases and 29,900 prostate cancer deaths. Measurement of serum PSA and palpation of the prostate gland remain the principal tools for prostate cancer screening. More than half of cancer patients with a PSA test of greater than 10 ng/mL have advanced disease. In contrast, most cancer patients with a PSA test of 4-10 ng/mL have early stage disease that is potentially curable. Unfortunately, at 4-10 ng/mL, the PSA test has a low specificity, since only 25% of these individuals have prostate cancer, and biopsy is required for disease diagnosis. Biopsy, in turn, has an expected false negative rate of 20%, necessitating rebiopsy of the large majority of men presenting with 4-10 ng PSA/mL serum. Detection of early prostate cancer requires many 'unnecessary' biopsies at high cost in terms of human stress and dollar expenditure.

A promising approach to the problem of large-scale rebiopsy for detection of early prostate cancer is based on work supporting the concepts of premalignant field disease and cancer-induced field effect. Premalignant field disease is defined as genetic and/or epigenetic modifications of morphologically normal cells that, as a consequence, have a markedly increased probability of neoplastic transformation. In response to continued exposure to carcinogenic insult, cells at multiple foci are initiated (defining a polyclonal disease) and undergo progression toward carcinoma of the prostate. In contrast, field effect is defined as the presence of reversible epigenetic changes in cells adjacent to tumor nodules, representing a response to altered signaling from the cancer cells. Cells within the premalignant field or the area of field effect exhibit a normal morphology but express phenotypic modifications that may be detected as qualitative or quantitative changes in expression of cellular proteins that include signaling, cytoskeletal, and cell adhesion proteins. Thus, it is possible to identify patients with a PSA of 4-10 ng/mL and early cancer cases, on the basis of a biopsy that is positive for 1) cancer or 2) in the event that the biopsy needle did not pass through a cancer nodule, field disease or field effect; *i.e.,* we propose that the combination of a PSA test of 4-10 ng/mL and a biopsy positive for field disease or field effect indicates the presence of early cancer and is the criterion for rebiopsy.

Using Quantitative Fluorescence Imaging Analysis (QFIA), our laboratory is searching for quantitative changes in selected proteins of epithelial cells in prostate tumor nodules, as potential biomarkers of early prostate cancer. The work is based on the hypothesis that field disease (or field effect) is relatively wide spread in a cancer-bearing prostate, permitting detection of prostate cancer with biopsies that do not include a sample of the cancer nodule. Thus, our work has two parts; 1) identification of persistent and characteristic quantitative changes of selected proteins in epithelial cells of cancerous acini, as potential biomarkers, and 2) evaluation of morphologically normal acini in cancer-bearing glands, for similar though possibly smaller quantitative changes in the same cellular proteins. Our reference standard for these determinations comprise age matched and time-of-collection matched BPH specimens.

During the past year, our laboratory has completed a preliminary evaluation of twenty-five candidates for quantitative biomarkers of prostate cancer. These included Tissue Transglutaminase (tTGase), Gelsolin, beta-Catenin (beta-CAT), Thymosin beta-4, G-Actin, E-Cadherin, Survivin, and purinergic receptor P_2X_7. We have focused on tTGase and beta-CAT, completing extensive studies quantifying phenotypic expression of these potential biomarkers in normal and cancerous prostate glands and cancer cell lines. Accomplishments during the past year are summarized in the sections that follow.

ACQUISITION OF TISSUE SPECIMENS

The laboratory has accrued 23 new cases of prostate cancer and three cases of BPH, during the past year. Tissue sections, cores, and fine needle aspirates (FNAs) were harvested systematically, from the peripheral zone (with some associated central zone) of each resected gland and fixed as described (*cf.* 'Fixation of Tissue Specimens'), embedded in paraffin or cryopreserved, archived, and logged into our database. In addition, our pathologists reviewed more than 200 archived cancer cases and BPH controls to identify 40 cancer cases and 40 matched controls. Cases and controls were matched on the basis of age, ethnicity, and year during which the tissues were archived.

FIXATION OF TISSUE SPECIMENS

Conditions of tissue fixation were evaluated for maximum and specific fluorescence labeling of standard tissue preparations including slide specimens of benign hyperplastic glands, an archived normal gland from an organ donor, and cancer cell lines. Conditions evaluated included the nature of the fixative, *i.e.*, cross-linking or non-cross-linking, and duration and temperature of fixation. The cross-linking fixatives tested were formalin, formaldehyde, and paraformaldehyde, and the non-cross-linking fixatives comprised Streck Fixative (zinc-based) and Methacarn (methanol/chloroform). In all cases, tissues were subsequently embedded in paraffin and single cells were stored in 25% ethanol at -80°C. Results differed to some degree on the basis of tissue and potential biomarker tested. Based on quantification of mean fluorescence intensity produced by labeling tTGase in acini of a non-cancer bearing prostate gland, fixation with Streck Fixative (room temperature, 24 hours) yielded the best results, followed closely by fixation with 5% formaldehyde (room temperature, 48 hours) and 4% paraformaldehyde (4°C, 24 hours) (**Figure 1**). However, a significantly lower proportion of prostatic acini were labeled after fixation with Streck Fixative (**Figure 2**). Results obtained with beta-CAT labeling differed from the data obtained with tTGase labeling. Acceptable fluorescence signal was obtained with all four fixatives tested (**Figure 3**). Based on mean fluorescence intensity, Streck Fixative again yielded the best results, followed closely by 5% formaldehyde. All acini were labeled under each of the fixation conditions.

We tested the compatibility of a number of fixatives including 1) Streck Fixative, 2) 4% paraformaldehyde, and 3) QFIA fixative (0.5% formaldehyde; 25% ethanol) on fluorescence labeling of beta-CAT in prostate cancer cell lines including the LNCaP cell line. On the basis of mean fluorescence intensity, all tested fixatives yielded acceptable labeling characteristics. Streck Fixative and QFIA fixative yielded comparable labeling whereas labeling with 4% paraformaldehyde was associated with reduced mean fluorescence intensity (**Figure 4**).

As a direct outcome of our studies of cell and tissue fixation for QFIA, we routinely fix prostate tissue fragments and cores in 5% formaldehyde, 4% paraformaldehyde (for cryosectioning), and Streck Fixative. Cell lines are routinely fixed in QFIA fixative and stored at -80°C, prior to imprinting to glass slides.

DEPARAFFINIZATION AND EPITOPE RECOVERY

Deparaffinization and epitope recovery with slide specimens of prostate tissue sections and cores have been fine tuned to reduce unintended effects on the specimens and variations in the conditions of each procedure. Slide specimens are deparaffinized with BioGenex dewaxing agent (Dewax) and the robotic Autostainer (BioGenex i6000). According to our original procedure, slides were exposed to four changes of Dewax for four minutes per change, and then

washed nine times with water and then two times with Optimax buffer. Monitoring the performance of the Autostainer, we found that slides near the end of the run tended to dry, when slides at the beginning of the run were being washed (9 washes per slide). To prevent drying of the specimens, slides are exposed to four changes of Dewax for three minutes per change and rinsed one time with water. Water-laden slides are transferred to slide trays containing water and washed with running tap water for 10 minutes. Subsequently, slides are transferred to Optimax buffer and soaked for 5 minutes. Epitope recovery is initiated by transferring the slides to citrate buffer (pH 6.0) at 95°C, in containers that are sealed to prevent evaporation and change of buffer ionic strength. Originally, containers were not sealed. After 25 minutes of incubation at 95°C, the sealed container is transferred to crushed ice and cooled for 30 minutes. Formerly, the container was opened to the room air and allowed to cool down, on the bench, for 2 hours; a procedure associated with measurable evaporation of the recovery buffer.

QUANTITATIVE FLUORESCENCE LABELING

Slide specimens of prostate glands and cell lines were labeled with streptavidin-fluorophore or secondary antibody-fluorophore conjugates, with a robotic Autostainer (Biogenex i6000). The standard protocol for labeling with streptavidin-Alexa (SA-Alexa) is presented in **Table '1'**. Originally, we labeled tissues with SA-Alexa 488, but switched to SA-Alexa 568 to

TABLE 1: Protocol for Robotic Labeling of Tissues and Cells with a Streptavidin-Fluorophore Conjugate, for Quantitative Fluorescence Imaging Analysis

PROTOCOL STEP	Time (minutes)	Buffer Rinses	Water Rinses	Incubations
Buffer	12	2	0	2
Serum Block	20	1	0	1
Avidin Block	15	5	0	1
Biotin Block	15	5	0	1
Primary Isotype Control or Primary Antibody	60	3	0	1
Biotinylated Secondary Antibody	30	3	0	1
Streptavidin Fluorophore	30	3	0	1
Buffer	12	2	0	2
Hoechst Dye	1	2	0	6

obviate 'spill' of fluorescence emission of Hoechst nuclear label into the target protein channel. For each targeted protein, we determined fluorescence emission with serial dilutions of primary antibody, secondary antibody, and SA-Alexa and generated emission *vs.* dilution plots that permitted identification of reagent dilutions required for quantitative analysis (**Figure 5**). Dilution within the 'saturation plateau' was selected for labeling. Saturation or stochiometric dilutions were 1/100, 1/100 and 1/100 for primary anti-beta-CAT, biotinylated secondary goat anti-mouse IgG, and SA-Alexa, respectively. Saturation dilutions for primary anti-tTGase, biotinylated goat anti-mouse IgG, and SA-Alexa were 1/200, 1/100 and 1/100, respectively (**data

not shown). The saturating dilution (1/100) of the secondary goat anti-mouse IgG-Alexa conjugate was the same as that of the biotin-conjugated secondary antibody.

An extensive review of the run logs of the Autostainer revealed incorporation of group batching into labeling runs that exceeded 12-14 slides. Batching involved holding groups of slides in buffer for *ca.* 2 hours typically at the beginning of the run, while other slides were processed. We tested the effects of batching on fluorescence signal, by measuring mean fluorescence intensity of standard BPH sections held in buffer for 2 hours, at different points in the labeling protocol. Depending on the location of batching, signal was reduced from a few percent to *ca.* 20% (**Figure 6**). The greatest reduction in signal was seen when buffering matched typical batching by the Autostainer. Since we anticipated the need to process up to 36 slides per run, batching would occur and seriously compromise the precision of our fluorescence measurements.

We determined that batching could be reduced or prevented by decreasing the number of steps in the labeling protocol, a condition that could be achieved by labeling cells with secondary antibody-Alexa conjugates rather than SA-Alexa conjugates. The standard protocol for labeling with secondary antibody-Alexa (SecAb-Alexa) is presented in **Table '2'.**

TABLE 2: Protocol for Robotic Labeling of Tissues and Cells with a Secondary Antibody-Fluorophore Conjugate, for Quantitative Fluorescence Imaging Analysis

PROTOCOL STEP	Time (minutes)	Buffer Rinses	Water Rinses	Incubations
Buffer	12	2	0	2
Serum Block	20	1	0	1
Primary Isotype Control or Primary Antibody	60	3	0	1
Secondary Antibody Alexa Conjugate	30	3	0	1
Buffer	12	2	0	2
Hoechst Dye	1	2	0	6

The run logs of 36-slide labeling runs were reviewed and revealed the absence of batching. Further, the specific fluorescence signal generated with SecAb-Alexa was comparable to that generated with SA-Alexa and exhibited a better signal:noise ratio, as exemplified by fluorescence labeling of Gelsolin in T24-61 bladder cancer cells (**Figure 7**).

Labeled slide specimens routinely are mounted in Prolong Gold (Molecular Probes) for quantitative fluorescence imaging. Originally, the specimens were mounted in n-propyl gallate (NPG). We found significant decay of the fluorescence signal from NPG mounted specimens both on repeated exposure to excitation energy and on storage at –20°C. In contrast, the signal from Prolong Gold mounted specimens displayed no significant decay with up to fifteen 20-second exposures to excitation energy and for up to five weeks of storage at –20°C.

INSTRUMENTATION ENHANCEMENT AND USE

Our Imaging/Image Analysis System consists of a fully automated Leica microscope with an eight-place Marzhauser stage, an 8/12 bit Hamamatsu CCD b/w camera, a Leica DFC480

color camera, and ImagePro Plus software. This past year, a major enhancement of the Fluorescence Imaging System was achieved by incorporation of a Hamamatsu Hg/Xe lamp. The enhancement was implemented by *ad hoc* modifications of the lamp housing, permitting coupling to the Leica Microscope Stand and effective focusing on the specimen field. The Hg/Xe lamp offers an excitation spectrum similar to that of the conventional Hg vapor lamp, but operates with remarkable stability and a life span of *ca.* 2000 hours. This contrasts with the unwanted flickering of the Hg vapor lamp that has a useful life span of *ca.* 100 hours. The Hg/Xe modification translates to significant enhancements in precision and reproducibility of our measurements.

Preceding each Imaging/Image Capture session, system performance is evaluated with fluorescence intensity beads expressing different emission energies. The session proceeds if the emission plot superimposes on previous plots. If a change is observed, system components will be evaluated and adjusted to achieve a return to the original performance standard, *i.e.*, emission plot.

EVALUATION AND IMPLEMENTATION OF KEY CONDITIONS FOR QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS (QFIA) OF POTENTIAL BIOMARKERS OF PROSTATE CANCER

QFIA offers the advantage of reproducible detection of small quantitative differences in the expression of marker proteins of interest. This point is illustrated in **Figure '8'**, depicting QFIA of BLCA-4 antigen in prostate and bladder cancer cell lines characterized by different levels of BLCA-4 expression. Cells were fixed in 0.5% formaldehyde in phosphate buffered saline for 15 minutes, diluted 1:1 with 50% ethanol, and stored at $-80\ ^\circ$C. Suspensions were thawed and imprinted to glass slides, and BLCA-4 antigen was labeled with SA-Alexa 568. To avoid slide batching by the Autostainer, individual labeling runs were completed with no more than 14 slides. **Figure '8'** documents reproducible discrimination ($p < 0.05$) of cells differing by *ca.* 25% in their expression of BLCA-4. Based on our experience with tissues and cell lines, we expect similar discrimination of potential biomarkers in tissue sections.

Our experimental design as exemplified by beta-CAT analysis incorporates three levels of quality control and reproducibility assessment. First, LNCaP cells are incorporated as a normalization and reproducibility standard. We evaluated inter- and intra-run and person-to-person reproducibility of measured fluorescence emission from LNCaP cells stoichiometrically labeled with SA-Alexa 568, a mouse monoclonal Ab specific for beta-CAT, and a secondary biotinylated goat anti-mouse IgG (**Figure 9**). Cells were imprinted immediately after thawing from $-80°$C, enhancing reproducibility within and among the slide sets. We found reproducibility compromised when fixed cells were removed from the $-80°$C freezer and allowed to sit on the bench for 30 or more minutes, prior to imprinting to microscope slides (**data not shown**). Using the ImagePro Plus software, we segmented cells by setting a lower intensity threshold just above background, *ca.* 200 on a 12-bit camera response range, and filtered out debris by setting a minimum threshold for event area. Typically, 200 to 500 cells are captured and analyzed. Individual slides in each set of three deviated from their mean by 5-11 % and means of inter-experimental sets varied 5-10%. Technologist-to-technologist reproducibility was also very good with means of triplicate slides differing by 2-5% within each experiment. The relatively large standard deviations (**Figure 9**) associated with the LNCaP cells reflect cells in different stages of the cell cycle rather than measurement imprecision.

As a second level of quality control, slide specimens of BPH glands are incorporated as a normalization and reproducibility standard. These slides are prepared with specimens collected from glands immediately after resection, and optimally fixed in 4% formaldehyde for 48 hours. In contrast to LNCaP cells, BPH specimens serve to control for variations in deparaffinization and epitope recovery. We evaluated inter- and intra-experimental reproducibility of measured fluorescence from slide preparations stoichiometrically labeled with SA-Alexa 568, mouse anti-beta-CAT, and biotinylated goat anti-mouse IgG (**Figure 9**). Prostatic acini were segmented, by setting a lower threshold just above background. Unwanted objects were filtered out, by setting a minimum feret (shortest caliper length). Typically, 100 or more acini are analyzed per slide preparation. Individual slides in each set of three deviated from their mean by 4-7 %, as reflected by their small standard deviations, and means of the inter-experimental sets varied by less than 10%.

Finally, for studies that require two or more labeling and imaging analysis runs, two pairs of matched cases and controls are carried over from each previous analysis, as a means of checking cross analysis reproducibility.

ANALYSIS OF TISSUE TRANSGLUTAMINASE EXPRESSION IN BENIGN HYPERPLASTIC AND CANCER-BEARING PROSTATE GLANDS, BY QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS (QFIA)

We first analyzed tTGase expression, in tissue sections from 20 pairs of matched cases and controls, with the dual purpose of completing a preliminary assessment of tTGase as a potential biomarker of prostate cancer and field disease (or field effect), and evaluating our analytical procedures. This study led to some of the procedural refinements described in previous sections (above). We measured mean fluorescence intensity of segmented acini as well as frequency of positively labeled acini in BPH glands, and normal and cancerous areas of cancer-bearing glands. The results with the first group of six matched cancer cases and controls are summarized in **Table '3'**. Mean fluorescence intensity was significantly reduced in

TABLE 3: Mean Intensity and Frequency of Labeled Acini in Benign Hyperplastic and Cancerous Prostate Glands

	BPH (6 cases)	Prostate Cancer (6 cases)		
		All Captured Acini	Cancerous Acini	Morphologically Normal Acini
Frequency of Labeled Acini (number of acini)	41% (589)	*17% (3532)	*#7% (2627)	#46% (905)
Mean Intensity of Labeled Acini (number of acini)	541 (272)	*421 (522)	*413 (166)	*424 (356)

* Significantly different from BPH cases, p<0.05; # Significantly different, p<0.05.

Mean Intensity of labeled acini in BPH glands and morphologically normal areas of cancer-bearing glands are statistically different (p < 0.01), and suggest a field effect or field disease.

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morphologically normal appearing acini of cancer bearing glands, in comparison to BPH glands, supporting a field effect or field disease and suggesting that quantitative changes in tTGase could be used to identify cancerous prostate glands with cores from morphologically normal areas, *i.e.*, with biopsies that did not include the cancer nodule(s). Unfortunately, a large fraction (up to 80%) of the acini in normal BPH glands do not label for tTGase, compromising the potential for reduced expression of this protein to serve as a biomarker for field effect or field disease in cancer bearing glands. This is illustrated in **Figure '10'** depicting adjacent, serial sections of a core biopsy from a resected BPH gland. **Panel 'A'** shows a section the nuclei of which are labeled with Hoechst dye, revealing prostatic acini, and **Panel 'B'** shows the neighboring serial section treated with SA-Alexa 488 for fluorescence labeling of tTGase. Only one acinus (white arrow) displayed tTGase labeling.

ANALYSIS OF BETA-CATENIN EXPRESSION IN BENIGN HYPERPLASTIC AND CANCER-BEARING PROSTATE GLANDS, BY QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS (QFIA)

In view of our finding that expression of tTGase is highly variable in normal BPH glands and often absent in a large proportion of acini in these glands, we turned our attention to phenotypic expression of beta-CAT as a potential biomarker of prostate cancer and field disease (or field effect). Beta-CAT is a high-level biomarker of cell-cell adhesion and a cell signaling protein. Based on our experience with tTGase, we first evaluated the pattern of beta-CAT expression in BPH specimens collected by our laboratory and optimally fixed with 5% formaldehyde. We found a bright, specific beta-CAT signal homogeneously distributed among acini of the slide specimens (**Figure 11**). **Panel 'A'** shows a section labeled with Hoechst nuclear dye, revealing prostatic acini, and **Panel 'B'** shows the neighboring serial section treated with SA-Alexa 568 for fluorescence labeling of beta-CAT. All of the acini express bright, specific labeling, a characteristic required for analysis of biomarkers in core biopsies.

We designed a QFIA study of six matched cancer cases and BPH controls. Slide specimens of paraffin-embedded tissue pieces were labeled with a combination of mouse monoclonal anti-beta-CAT, biotinylated goat anti-mouse IgG, and SA-Alexa 568. Fluorescence images were captured and quantitatively analyzed with ImagePro Plus software. Mean fluorescence intensity (MFI; mean pixel value in each segmented acinus, corrected for background of the primary antibody isotype control) was determined for 100 or more acini of each BPH gland and for morphologically normal and cancerous areas of each cancer-bearing gland (**Figure 12**). In relation to MFI of the BPH acini, MFI was significantly reduced in cancerous acini ($p < 0.05$) but not in morphologically normal acini ($p = 0.28$) of cancer-bearing glands. A threshold set at MFI=175 yields a specificity of 83% and a sensitivity of 67% or 83% based on morphologically normal (MN) or cancerous (Ca) acini, respectively. Some points in the scatter plot are very close to this threshold, indicating that small changes in MFI measurements can move points to the opposite side of the threshold and change specificity and sensitivity.

QFIA clearly differentiated BPH and cancerous areas of cancer-bearing glands and indicated a trend toward reduced beta-CAT expression in morphologically normal areas of cancerous glands (**Figure 12**); consequently, we extended our studies to include an additional fourteen matched cancer cases and BPH controls. Again, MFI of the Ca acini was significantly reduced ($p < 0.01$) in comparison to MFI of the BPH acini, but the trend toward reduced MFI of

the MN acini was no longer apparent (**Figure 13**). The results are associated with a marked increase in the range of MFI measurements of both BPH and Ca acini and a marked upward shift in the MFI of each group, in comparison to the first set of six matched cases and controls (**Figure 12**). These observations prompted extensive review and significant revisions of our fluorescence labeling procedures, as described in the previous sections of this report. In addition, we reviewed H&E slides of selected archived blocks used in this study and found evidence of tissue decomposition in at least one archived specimen, prompting routine evaluation of H&E slides for subtle and degenerative morphological changes in archived specimens screened for study. It remains possible that beta-CAT may not serve as a cancer biomarker in core biopsies that miss the cancer nodule(s), either because this protein is not down regulated in morphologically normal areas of cancerous glands or because its down regulation is insufficient to be detected against measurement variances associated with poorly controlled collection and fixation of archived specimens. We are now initiating a revised program of analysis of beta-CAT expression in prostate cancer, incorporating significant procedural improvements aimed at maximizing precision and reproducibility of our fluorescence measurements, and consequently will be able to address these considerations.

REPORTABLE OUTCOMES

Abstracts

1. Dali Huang, George P. Casale, Nizar K. Wehbi, George P. Hemstreet, III. Tissue Transglutaminase Down-regulation: a Potential Biomarker for Prostate Cancer Premalignancy. American Association for Cancer Research (AACR), January 25-29, 2004, Waikoloa, Hawaii.

2. George P. Casale, Nizar K. Wehbi, Dali Huang, Chantey Morris, George P. Hemstreet, III. A Monoclonal Antibody for Macromolecular Adducts of Estradiol-3,4-quinone, a Suspected Initiator of Breast and Prostate Cancers. American Association for Cancer Research (AACR) 59th Annual Meeting, March 27-31, 2004, Orlando, Florida.

CONCLUSIONS

During this past year, our research group has completed 1) preliminary evaluations of twenty-five candidates for quantitative biomarkers of prostate cancer, 2) extensive quantitative proteomic analyses of tissue transglutaminase and beta-Catenin in normal and cancerous prostate glands, and 3) extensive reviews and revisions of our quantitative fluorescence labeling procedures. Our goal is to detect prostate cancer in those individuals whose serum PSA is 4-10 ng/mL and whose initial biopsy missed the cancer nodule(s). Our working hypothesis is that quantitative changes in high-level phenotypic biomarkers occur in relatively expansive areas of morphologically normal acini and are indicative of a field disease or field effect associated with the presence of prostate cancer. The work represents, for our laboratory, a transition from implementing and fine-tuning the most current QFIA technologies to their application in human studies aimed at translating information regarding the molecular biology of prostate cancer and carcinogenesis into sensitive and specific approaches to early cancer detection.

Our studies of tissue transglutaminase demonstrated significant down regulation of protein expression in morphologically normal acini of cancer-bearing glands in comparison to normal acini of benign hyperplastic glands, providing some evidence in support of the concepts of field disease and field effect. However, the markedly heterogeneous distribution of tissue transglutaminase disqualified the protein as a cancer biomarker in core biopsies that do not include samples of the cancer nodule(s). Turning our attention to other potential biomarkers, we found beta-Catenin to be a candidate of interest because of its bright, specific, and homogeneous labeling of acini in non-cancer-bearing prostate glands. Our work has demonstrated a significant reduction of beta-Catenin in cancerous acini compared to normal acini of BPH glands, suggesting that quantitative down regulation of this protein may be used to detect field disease or field effect. In fact, our first analysis of beta-Catenin expression in six archived prostate cancer cases and six age-matched BPH controls indicated a trend for reduced expression in the normal acini of cancer-bearing glands. An extension of this analysis to an additional fourteen matched cases and controls produced results that were not consistent with the earlier data, and prompted extensive reviews and revisions of quantitative fluorescence labeling procedures (detailed in this report). These significant enhancements in the precision and reproducibility of our QFIA measurements will allow us to make a decisive evaluation of beta-Catenin as a quantitative biomarker of prostate cancer.

It remains possible that beta-CAT may not serve as a cancer biomarker in core biopsies that miss the cancer nodule(s), either because this protein is not down regulated in morphologically normal areas of cancerous glands or because its down regulation is insufficient to be detected against measurement variances associated with poorly controlled collection and fixation of archived specimens. We are now initiating a revised program of QFIA of beta-CAT expression in prostate cancer, incorporating significant procedural improvements aimed at maximizing precision and reproducibility of our measurements, and consequently we will be able to address these considerations.

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FIGURE 1: EFFECT OF DIFFERENT FIXATION CONDITIONS ON MEAN FLUORESCENCE INTENSITY OF LABELED TISSUE TRANSGLUTAMINASE IN ACINI OF A NON-CANCEROUS PROSTATE GLAND
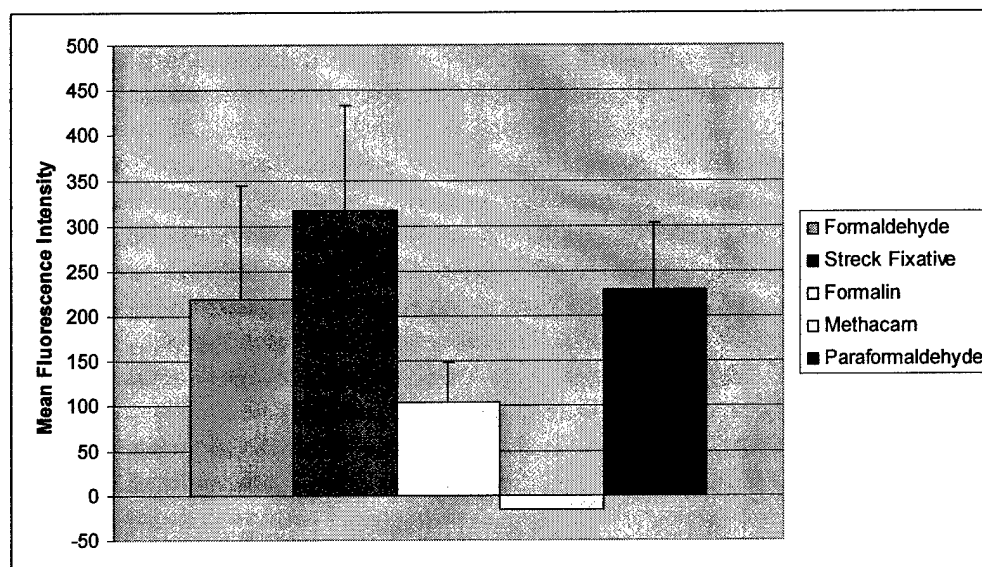

FIGURE 2: EFFECT OF DIFFERENT FIXATION CONDITIONS ON THE FREQUENCY OF ACINI POSITIVELY LABELED FOR TISSUE TRANSGLUTAMINASE IN TISSUE SECTIONS OF A NON-CANCEROUS PROSTATE GLAND
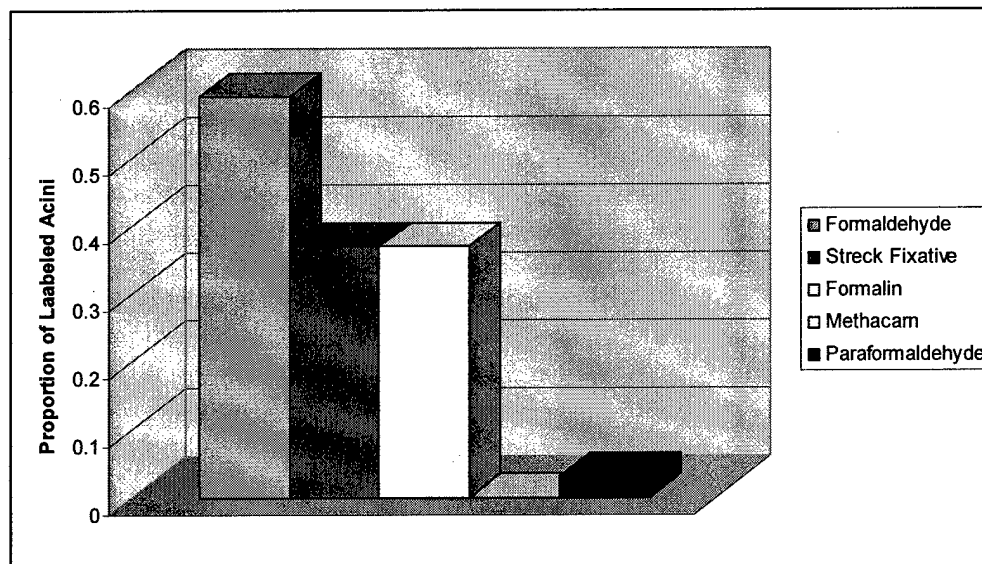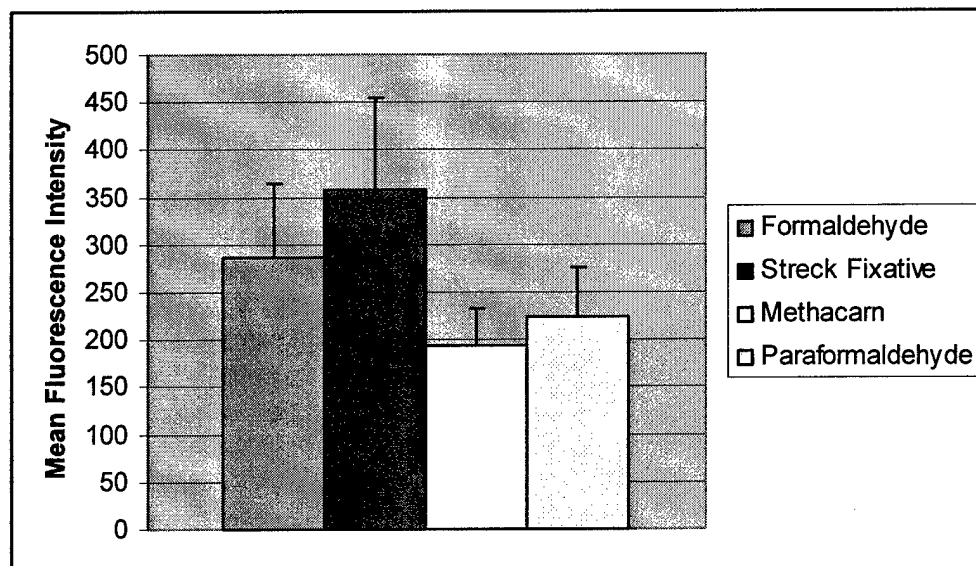
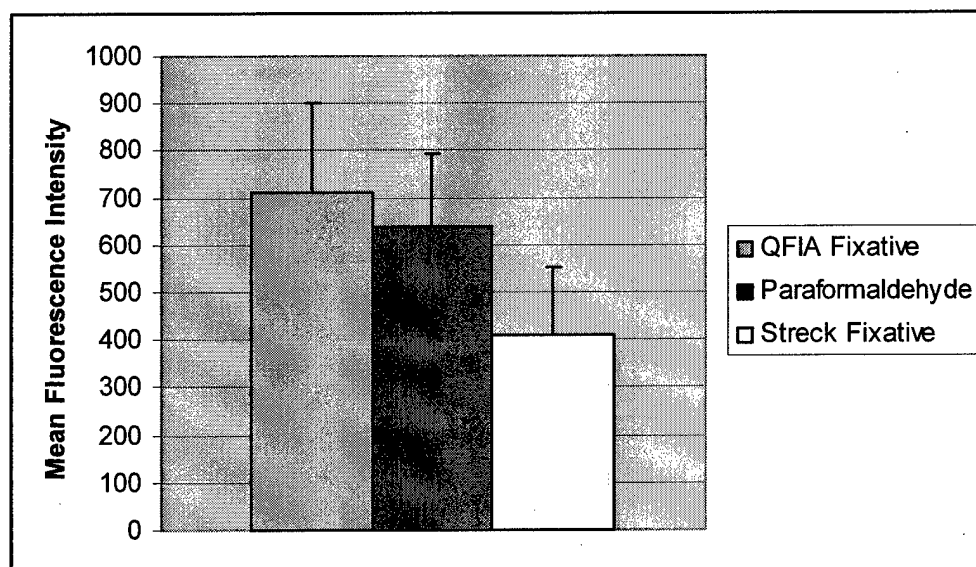## FIGURE 3: EFFECT OF DIFFERENT FIXATION CONDITIONS ON MEAN FLUORESCENCE INTENSITY OF LABELED BETA-CATENIN IN ACINI OF A NON-CANCEROUS PROSTATE GLAND

FIGURE 4: EFFECT OF DIFFERENT FIXATION CONDITIONS ON MEAN FLUORESCENCE INTENSITY OF LABELED BETA-CATENIN IN LNCAP CELLS

FIGURE 5: DILUTION OF PRIMARY AND SECONDARY ANTIBODY AND STREPTAVIDIN ALEXA (SA ALEXA) PREPARATIONS TO DETERMINE SATURATING DILUTIONS (Arrows) FOR QFIA
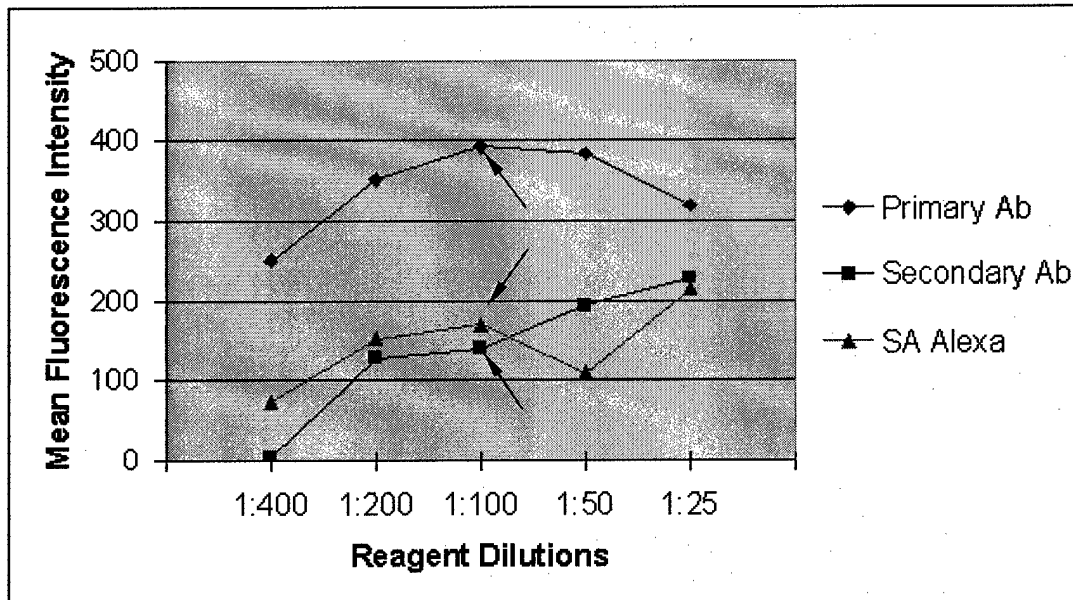
FIGURE 6: EFFECT OF AUTOSTAINER BATCHING ON THE EFFICIENCY OF LABELING BETA-CATENIN IN NORMAL PROSTATE TISSUE SECTIONS, FOR QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS
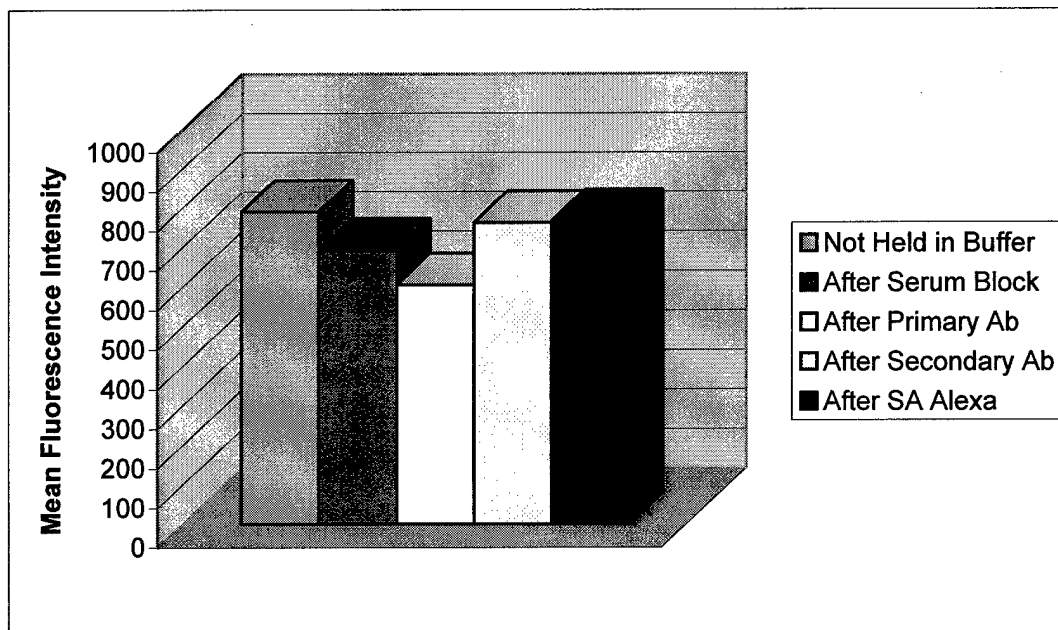
FIGURE 7: T24-61 CELLS LABELED WITH A PRIMARY ANTIBODY TO GELSOLIN AND EITHER A BIOTINYLATED SECONDARY ANTIBODY IN COMBINATION WITH STREPTAVIDIN ALEXA OR A SECONDARY ANTIBODY ALEXA CONJUGATE
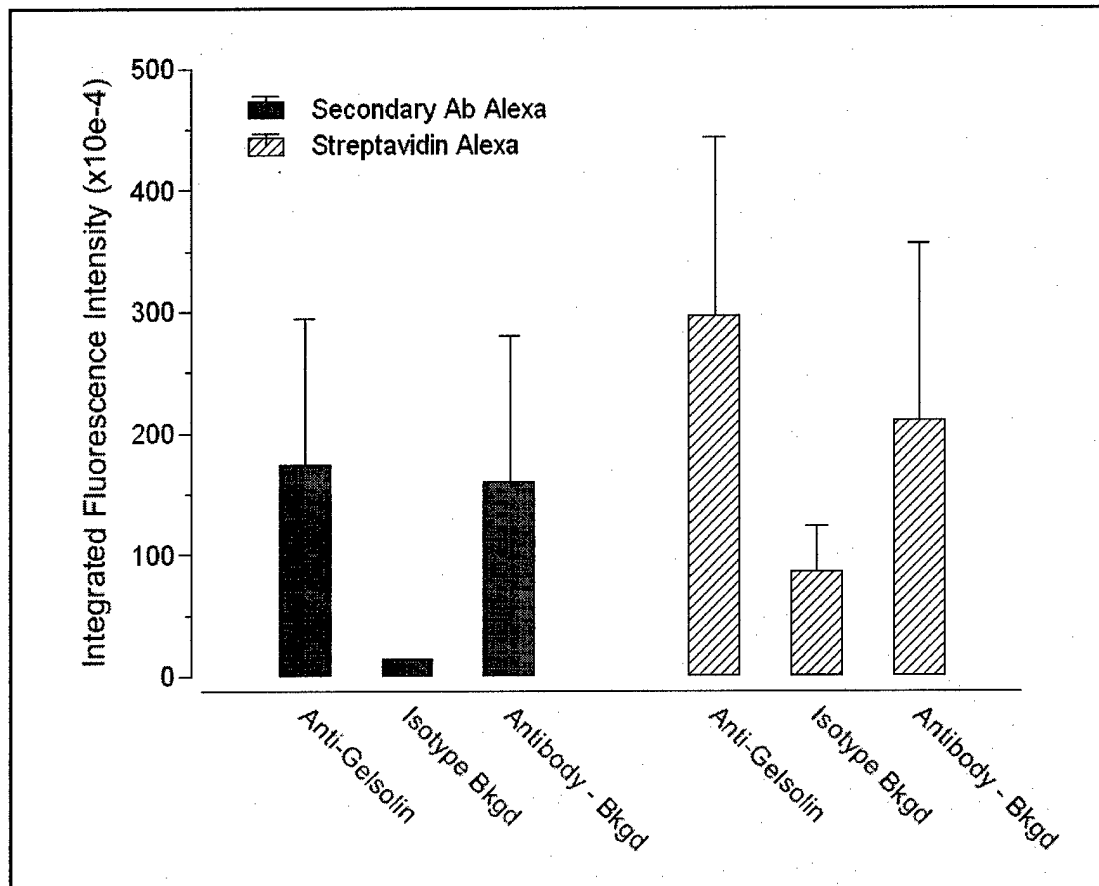
FIGURE 8: QUANTITATIVE DISCRIMINATION OF BLCA-4 DIFFERENTIALLY EXPRESSED IN SELECTED CELL LINES, BY QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS
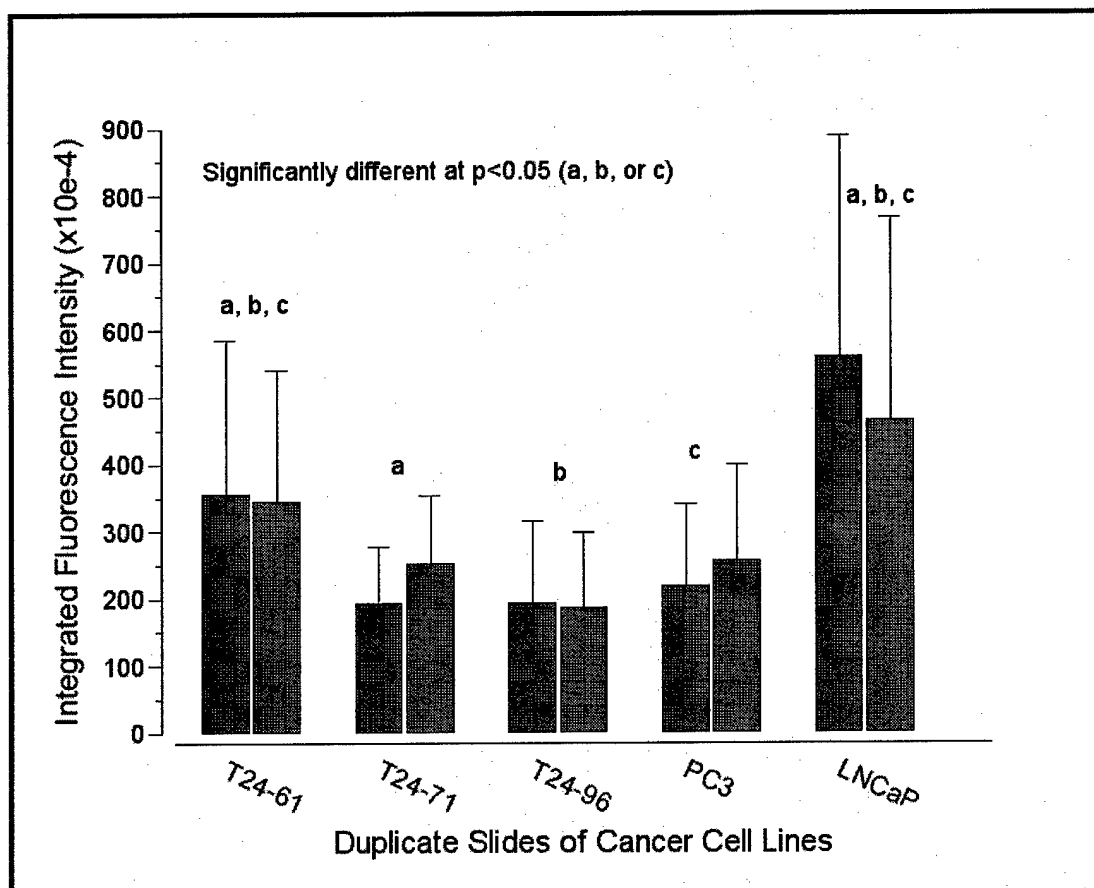
FIGURE 9: BETA-CATENIN EXPRESSION IN A PROSTATE CANCER CELL LINE (LNCaP) AND SECTIONS OF A BENIGN HYPERPLASTIC PROSTATE GLAND (BPH), MEASURED BY QUANTITATIVE FLUORESCENCE IMAGING ANALYSIS: INTRA- AND INTER-EXPERIMENT REPRODUCIBILITY
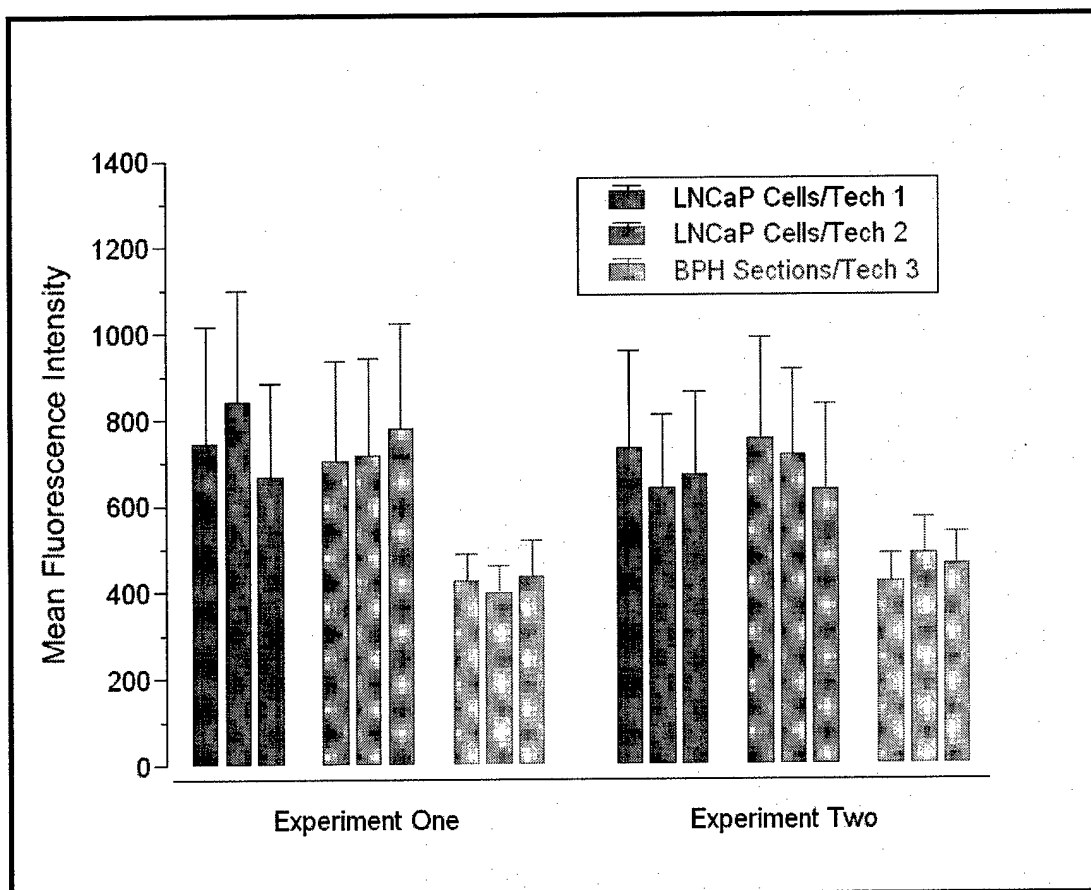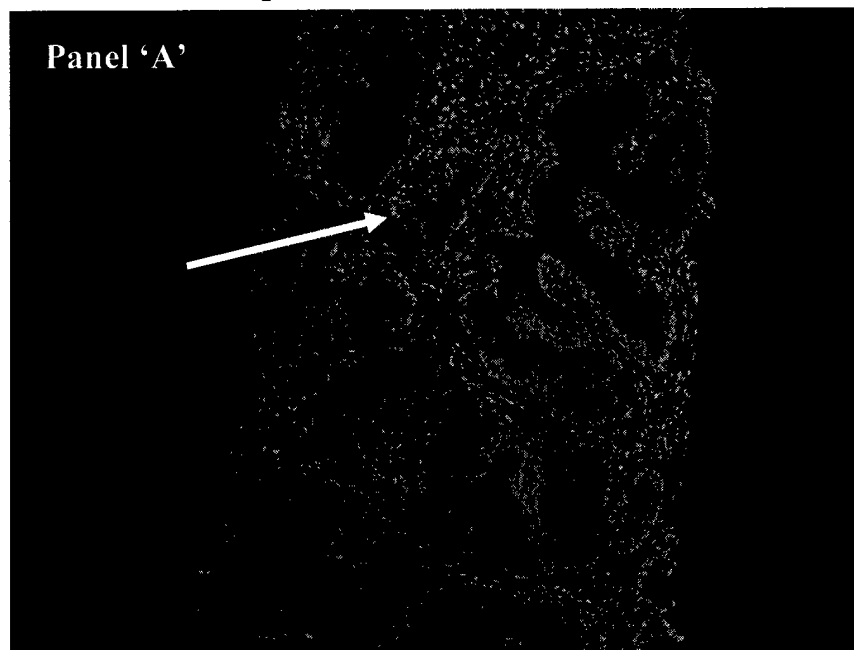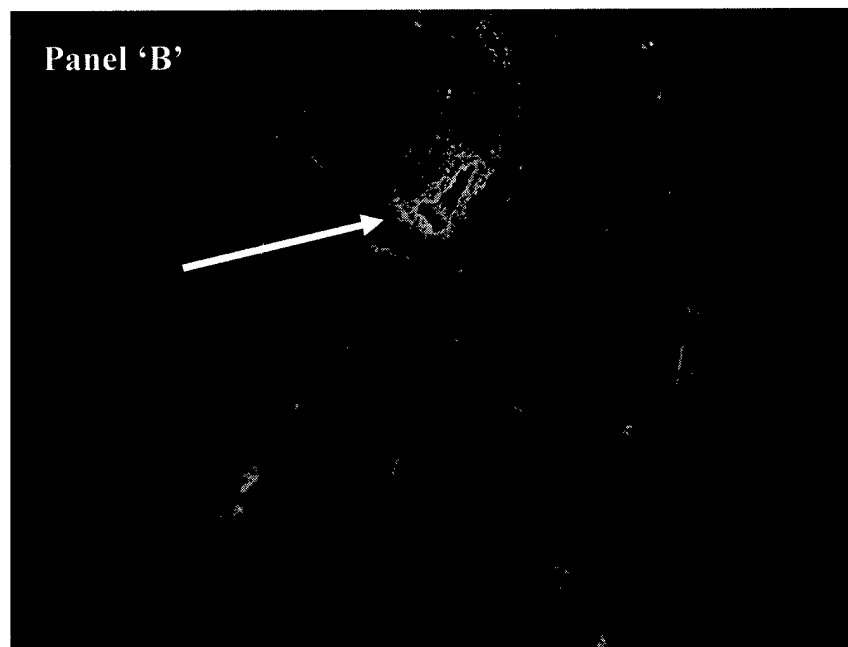
FIGURE 10: Heterogeneous Expression of Tissue Transglutaminase among Normal Acini in Core Biopsies of a Resected BPH Gland
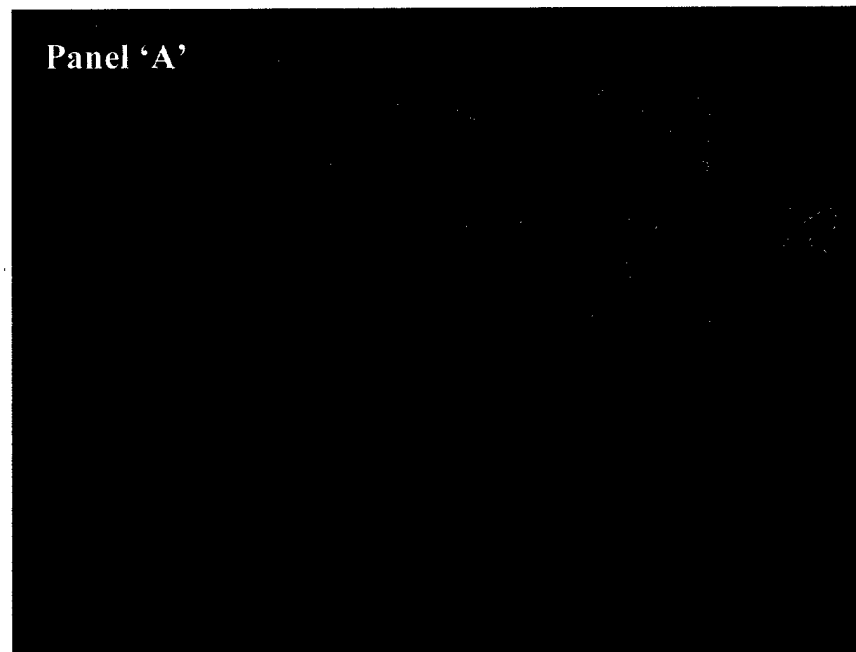

Panel 'A'

Panel 'A': Core biopsies were collected by our laboratory and optimally fixed in 5% formaldehyde. Slide specimens were labeled with Hoechst nuclear dye, revealing prostatic acini.
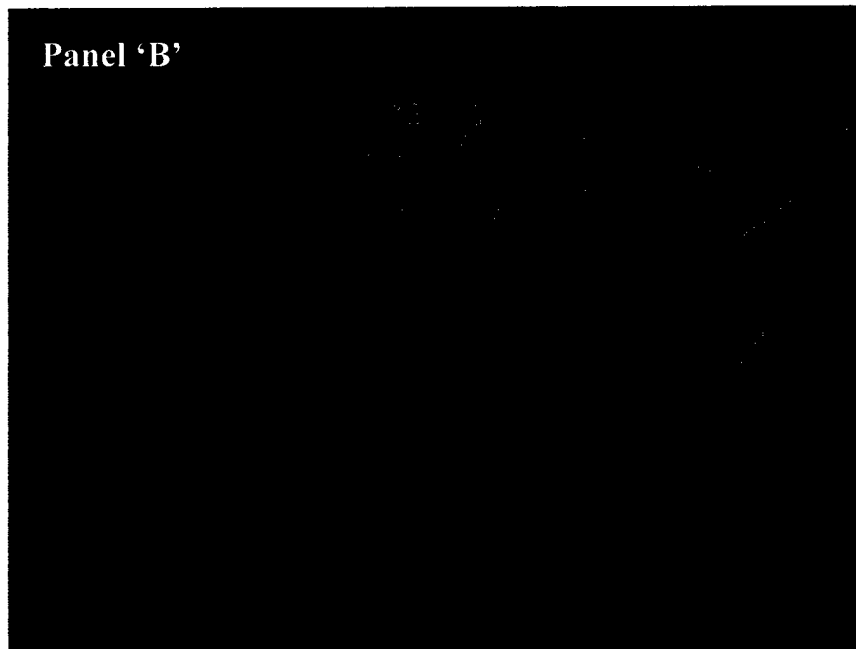

Panel 'B'

Panel 'B: Neighboring serial sections of the same core (Panel 'A') were processed for QFIA of tissue transglutaminase (tTGase), with a mouse monoclonal anti-tTGase antibody, secondary goat antibody, and a streptavidin-Alexa 488 conjugate. One acinus is labeled (arrow).

FIGURE 11: Bright, Specific and Homogeneous Expression of Beta-Catenin among Normal Acini of BPH Glands

Panel 'A': Slide specimens of a BPH gland were labeled with Hoechst nuclear dye, revealing the prostatic acini.

Panel 'B': Neighboring serial sections of the same BPH gland (Panel 'A') were processed for QFIA of beta-Catenin, with a mouse anti-beta-Catenin antibody, secondary goat antibody, and a streptavidin-Alexa 568 conjugate. All acini are labeled.

FIGURE 12: Differential Expression of Beta-Catenin in Acini of Benign Hyperplastic Prostate Glands (BPH) and Morphologically Normal (MN) and Cancerous (Ca) Areas of Cancer Bearing Glands
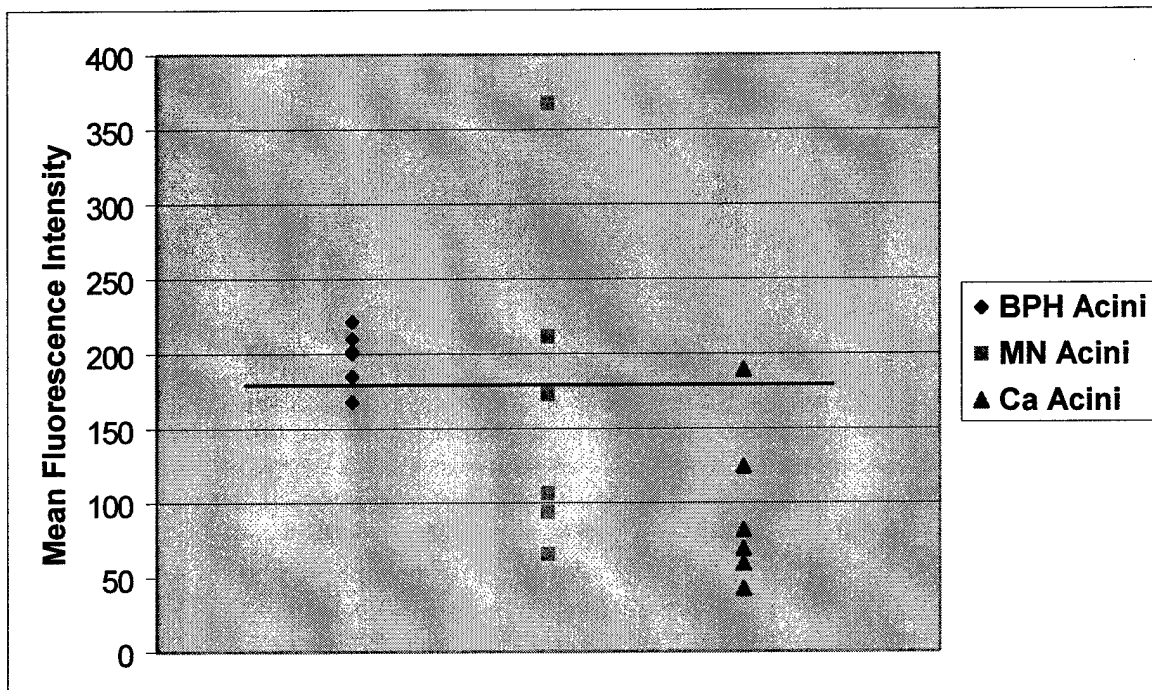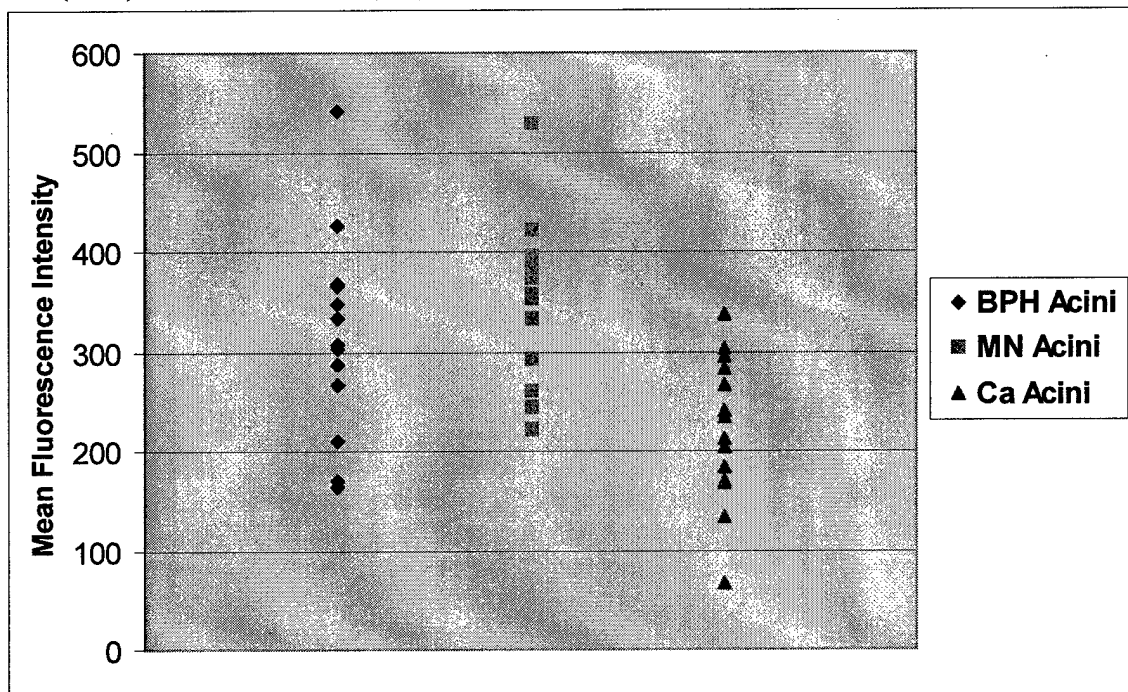
FIGURE 13: Differential Expression of Beta-Catenin in Acini of Fourteen Benign Hyperplastic Prostate Glands (BPH) and Morphologically Normal (MN) and Cancerous (Ca) Areas of Fourteen Cancer Bearing Glands

DAMD17-02-1-0121: Biomarker Based Individual Risk Assessment for Prostate Cancer
George P. Hemstreet III, MD, PhD

Abstract presented in AACR Meeting, January 25-29, 2004, Waikoloa, Hawaii

Authors: Dali Huang , George P. Casale, Nizar K. Wehbi, George P. Hemstreet III

Tissue Transglutaminase Down-regulation: a Potential Biomarker for Prostate Cancer Premalignancy

Previous studies indicate that there are differences in the expression of tissue transglutaminase (tTGase) in prostate cancer tissues when compared to benign prostatic hyperplasia (BPH) utilizing image analysis and conventional immunohistochemical staining.[1,2] In these studies, there was also a visual down regulation of tTGase in PIN lesions and a suspected down regulation of tTGase in the normal appearing glands of cancer-bearing prostates. Based on the concept of biochemical field disease, we previously demonstrated that field changes can predict the development of bladder cancer 3-5 years prior to clinically manifest disease, utilizing quantitative fluorescence image analysis (QFIA).[3,4] In order to further discriminate subtle down regulation of this biomarker in the normal appearing cells in the premalignant field and as a first step towards developing a male PAP test, we quantified the expression of tTGase in archival paraffin-embedded tissues from 6 cases of prostate cancer and 6 cases of BPH.

Materials and Methods: Age-matched archived tissue blocks (6 cases of BPH and 6 cases of prostate cancer) were obtained from the department of Pathology/Microbiology at the University of Nebraska Medical Center. Tissue sections were deparaffinized, re-hydrated, processed for antigen retrieval, and stoichiometrically labeled for tissue transglutaminase with a specific monoclonal antibody in conjunction with a biotinylated secondary antibody and streptavidin-AlexaFluor®488. Fluorescence images were captured and analyzed by a Leica automated microscope system and Image-Pro Plus software. Differences between group means were statistically evaluated by Student's t-Test.

Results: The frequency of labeled glands was higher in the BPH specimens (41%) compared to the cancer specimens (17%; $P<0.05$). Within cancer-bearing glands, labeling frequency was significantly higher ($P<0.05$) in the normal-appearing areas (46%) compared to cancerous areas (7%). There was no difference between BPH specimens and normal-appearing areas of the cancer specimens. Mean labeling intensity (540) of positive glands in the BPH specimens was elevated in comparison with labeling (420) of positive glands in the cancer cases, but did not achieve statistical significance ($P=0.21$). There was also no statistical difference between normal-appearing and cancerous areas of cancer-bearing glands. The mean intensity of all positive BPH glands (342 from 6 cases) was greater than that of all positive glands in either the normal-appearing (625 glands) or cancerous areas (236 glands) of the 6 cancer cases ($P<0.001$). There was no difference, however, between the normal-appearing and cancerous areas of cancer-bearing glands.

Conclusions: These preliminary results with age-matched prostate cancer and BPH cases support the concept of field effect or field disease in normal-appearing glandular tissue in cancer-bearing prostates, and the application of QFIA to single-cell proteomics to

DAMD17-02-1-0121: Biomarker Based Individual Risk Assessment for Prostate Cancer
George P. Hemstreet III, MD, PhD

establish a biomarker profile that identifies patients with an initially negative prostate biopsy, who require rebiopsy. This biomarker profile could be used to determine the risk of developing biologically active prostate cancer, detect early disease, and monitor effectiveness of chemopreventive agents. 1) Cancer, **89:** 412, 2000. 2) J Urol, **167:** 2215, 2002. 3) J Natl Cancer Inst, **93:** 427, 2001. 4) Proc Natl Acad Sci U S A, **90:** 8287, 1993

DAMD17-02-1-0121: Biomarker Based Individual Risk Assessment for Prostate Cancer
George P. Hemstreet III, MD, PhD

Abstract submitted to the AACR, March 27-31, 2003, Orlando, Florida

A MONOCLONAL ANTIBODY FOR MACROMOLECULAR ADDUCTS OF ESTRADIOL-3,4-QUINONE, A SUSPECTED INITIATOR OF BREAST AND PROSTATE CANCERS

George P. Casale, _Nizar K. Wehbi, Dali Huang, Chantey Morris, George P. Hemstreet III_. University of Nebraska Medical Center, Omaha, NE

Epidemiology and laboratory animal studies have established a linkage between breast and prostate cancers and estrogen exposure. Mammalian tissues metabolize 17-beta-estradiol (E_2) to the catechols 2-hydroxy E_2 (2-OHE$_2$) and 4-hydroxy E_2 (4-OHE$_2$). Recent studies support a close association between preferential 4-hydroxylation of E_2 and estrogen dependent carcinogenesis (E_2-DC). In model systems, organs susceptible to E_2-DC express estrogen 4-hydroxylase (CYP1B1) and produce more 4-OHE$_2$ than 2-OHE$_2$. Organs resistant to E_2-DC do not express CYP1B1 and produce predominantly 2-OHE$_2$. Catechol estrogens can be oxidized to quinones that bind covalently to DNA and proteins. The present work is based on the hypothesis that tissues engaged in E_2-DC, produce E_2-3,4-quinone (E_2-3,4-Q) in amounts sufficient to escape detoxification and form DNA and protein adducts that participate in carcinogenesis. Further, E_2-3,4-Q adducted to cellular proteins may serve as a biological marker of "escaped" E_2-3,4-Q and risk of both breast and prostate cancers.
We have developed a monoclonal antibody (Mab) with high specificity and affinity (K_a= 0.5 x10^8 M^{-1}) for E_2-3,4-Q adducted to any cellular macromolecule.
Immunohistochemical staining with the antibody produced intense, specific labeling in slide preparations of rat breast tissue treated with E_2-3,4-Q, no detectable labeling of tissues treated with E_2-2,3-Q and little or no background. In addition, the antibody produced a specific fluorescence signal in PC3 prostate cancer (PC) cells exposed _in vitro_ to 4-OHE$_2$ (nominal 10 micromolar), but no signal in similarly treated DU-145 PC cells. In conclusion, this newly developed antibody may serve as a unique and sensitive probe to assess 1) a plausible mechanism of breast and prostate carcinogenesis, and 2) the E_2-3,4-Q-protein adduct as a biomarker for both breast and prostate cancer risk. Funded by the U.S. Department of Defense.